AD_____

Award Number: DAMD17-01-1-0274

TITLE: NF-κB-Mediated Repression of GADD153/CHOP: A Role in Breast Cancer Initiation

PRINCIPAL INVESTIGATOR: Harikrishna Nakshatri, Ph.D.
Hui Lin Chua, Ph.D.

CONTRACTING ORGANIZATION: Indiana University
Indianapolis, Indiana 46202-5167

REPORT DATE: August 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Annual (1 Aug 01 - 31 Jul 02)

4. TITLE AND SUBTITLE
NF-κB-Mediated Repression of GADD153/CHOP: A Role in Breast Cancer Initiation

5. FUNDING NUMBERS
DAMD17-01-1-0274

6. AUTHOR(S)
Harikrishna Nakshatri, Ph.D.
Hui Lin Chua, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Indiana University
Indianapolis, Indiana 46202-5167

E-Mail: hnakshat@iupui.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Factors that control initiation of breast cancer are largely unknown. Mammary epithelial cells usually have a limited life span, which is governed by a balance between pro-survival and pro-apoptotic proteins. Imbalance in the expression level of these proteins, particularly pro-survival factors, is believed to promote immortalization and transformation of mammary epithelial cells. The transcription factor NF-κB can alter the ratio between pro-survival and pro-apoptotic proteins as it can induce the expression of several pro-survival genes and decrease the expression of pro-apoptotic genes. Our studies have shown that NF-κB upregulates pro-survival genes TRAF-1, cIAP-2 and Mn-SOD and inhibits the expression of pro-apoptotic gene GADD153/CHOP. In this award, we are investigating whether NF-κB mediated repression of GADD153 expression plays any role in breast cancer initiation upon DNA damage by environmental toxicants. Our approach is to overexpress the NF-κB subunit p65 in immortalized mammary epithelial cell line MCF-10A and evaluate transformation of these cells by environmental toxicants such as methyl methanesulfonate. Soft agar and matrigel assays will be used to measure transformation. Our initial attempts to obtain MCF-10A cells overexpressing the p65 subunit using plasmid-based vectors was not successful. Therefore, we have generated retrovirus that can transduce p65 to MCF-10A cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, cancer biology, apoptosis, environmental toxicants	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION:

Understanding the mechanism of breast cancer initiation is critical for developing any chemoprevention strategies. Normal mammary epithelial cells from which cancer usually originate have limited life span. Immortalization is the first step that leads to continuous growth of mammary epithelial cells (1). Cell cycle regulatory, anti-apoptotic and pro-apoptotic proteins play a significant role in immortalization process (1). Immortalized cells attain cancerous growth properties (transformation) due to additional mutations that lead to either loss of tumor suppressor genes or activation of oncogenes. The transcription factor NF-κB promotes both immortalization and transformation by controlling the expression of cell cycle regulatory, anti-apoptotic and pro-apoptotic genes (2, 3). NF-κB is usually sequestered in the cytoplasm of resting cells through its association with inhibitor of kappaB proteins and translocates to nucleus upon exposure of cells to cytokines and growth factors (3). NF-κB then binds to response elements and induces the expression of genes involved in invasion, metastasis and chemotherapy resistance. We and others have shown that NF-κB is constitutively active in breast cancer and is responsible for overexpression of several anti-apoptotic genes as well as repression of the pro-apoptotic gene GADD153/CHOP (4-7). GADD153 is induced when DNA is damaged or cells are under stress. Depending on the extent of damage, cells either repair DNA damage and survive or die. GADD153 is believed to promote death of cells with severe damage, thereby limiting accumulation of cells with mutations. Thus, GADD153 is likely to play a role in preventing breast cancer initiation. Because NF-κB reduces GADD153 expression, it is possible that cells that contain constitutively active NF-κB will survive after DNA damage. Such cells with damaged DNA are more prone for transformation. This award is to test this possibility. There are three aims in this proposal: 1) To determine whether inhibition of GADD153 by NF-κB is essential for survival and/or transformation of MCF-10A cells when exposed to MMS or grown under nutrient-deprived condition. 2) To determine whether inhibition of GADD153 by NF-κB leads to altered activity of the transcription factor C/EBPβ and differentiation of MCF-10A cells. 3) To determine the influence of p53 on the anti-apoptotic function of NF-κB in MCF-10A cells grown under nutrient-deprived condition or exposed to MMS.

BODY:

As per statement of work, following studies of specific aim I were part of year 1:
Months 1-5: establish MCF-10A cells overexpressing p65NLS50 or ras and characterize them with respect to constitutive NF-κB activity.
Months 6-8: Determine MMS-inducible and nutrient-deprivation inducible expression of GADD153 in MCF-10A, ras and p65NLS50 cells by Northern and Western blots. Determine apoptosis by annexin V labeling, PARP cleavage and DNA laddering.

Months 9-15: Determine the susceptibility of MCF-10A, ras and p5NLS50 cells to MMS-induced transformation.

Results: Not all the work included in statement of work could be completed in year one because of delay in recruitment of post-doctoral fellow. However, following studies have been completed in year one, which should lead to more productive second year.

Recent studies indicate that NF-κB subunits p50 and c-rel in addition to p65 are overexpressed in breast cancer (5, 6). In addition, the activity of IKK complex, which is involved in NF-κB activation, is elevated in breast cancer (8). To ensure that repression of GADD153 is mediated mostly by the p65 subunit of NF-κB, we performed transient transfection assay in MDA-MB-231 breast cancer cells with GADD153 promoter-CAT reporter and various subunits of NF-κB. Two reporter constructs, one containing –954 to +91 and the other with –247 to +91 region of GADD153 were used. The p65 subunit but not p50 or c-rel inhibited GADD153 promoter activity. Among the upstream kinases involved in NF-κB activation, only IKKα subunit inhibited the activity of GADD153 promoter. These results indicate that 1) the p65 subunit is most efficient in inhibiting GADD153 promoter activity; 2) sequences responsible for p65-mediated repression are located within –247 to +91.

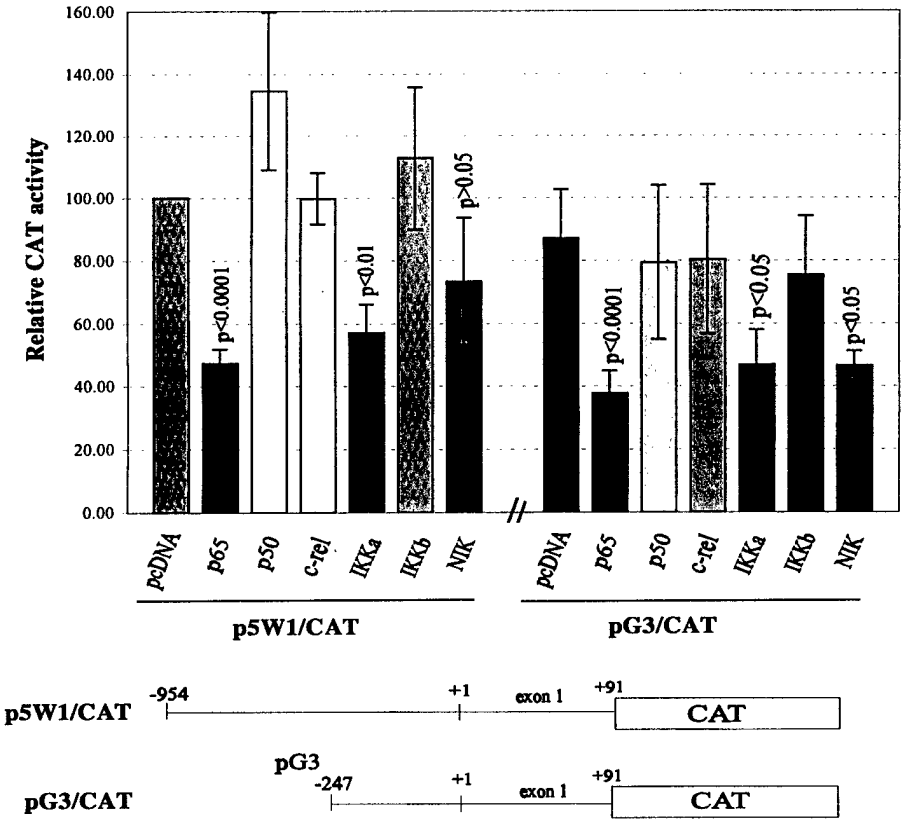
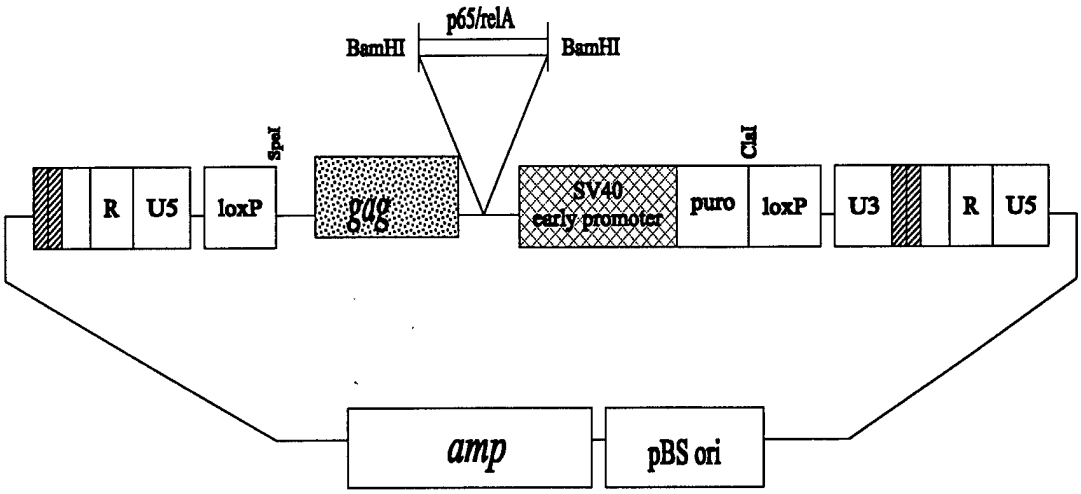


Fig. 1: The effect of various NF-κB subunits on GADD153 promoter activity.

We attempted to generate MCF-10A cells overexpressing p65. Because the p65 subunit can induce inhibitor of kappaB (IκBα), which can sequester p65 in the cytoplasm, we made a variant of p65 that binds less efficiently to IκBα (p65NLS50). Because the original vector that codes for p65NLS50 lacks any selection marker, we cotransfected p65NLS50 with pcDNA3 vector that confers resistance to G418. However, even after many attempts, we did not obtain any p65 overexpressing colonies. To overcome this difficulty, we have now generated two types of vectors that can code for p65NLS50. In one of them, we have directly cloned p65NLS50 cDNA into pcDNA3 vector. The p65NLS50 has also been cloned into a modified pBaBe-puro vector (see below). In this vector, p65NLS50 is flanked by loxP sequences, which will allow conditional deletion of p65NLS50 using cre-recombinase. Retrovirus is currently being generated and should be available for infecting MCF-10A cells in two weeks.



KEY RESEARCH ACCOMPLISHMENTS:

- The p65 subunit of NF-κB is the major repressor of GADD153/CHOP
- Sequences required for repression are located within –247 to +91 of GADD153
- Generated a retrovirus construct that permits conditional overexpression of p65 in MCF-10A cells.

REPORTABLE OUTCOMES:

None

CONCLUSIONS: In year one, we have been able to identify the NF-κB subunit responsible for repression of GADD153 and to localize the region of the promoter essential for repression. There is a possibility that repression is mediated by sequences in

the exon 1 (Fig. 1), which is included in our reporter constructs. We are examining this possibility by generating additional mutants lacking exon 1. We have also generated additional constructs where the sequences of exon 1 have been transferred to RSV/CAT reporter. If the sequences of exon 1 are responsible for repression, p65 should repress the expression of this chimeric RSV/CAT. These studies will help us in elucidating the mechanism of repression by p65.

We have created retrovirus that codes for p65 and transduced p65 can be deleted from cells at any time. This technique will allow us to examine whether transient overexpression of p65 is sufficient for transformation.

REFERENCES:

1. Wright, W. E. and Shay, J. W. Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology, Nat Med. *6:* 849-51., 2000.
2. Ghosh, S. and Karin, M. Missing pieces in the NF-kappaB puzzle, Cell. *109 Suppl:* S81-96., 2002.
3. Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. NF-kappaB in cancer: from innocent bystander to major culprit, Nature Rev Cancer. *2:* 301-10., 2002.
4. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth, Mol Cell Biol. *17:* 3629-39, 1997.
5. Sovak, M. A., Bellas, R. E., Kim, D. W., Zanieski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer, J Clin Invest. *100:* 2952-60, 1997.
6. Cogswell, P. C., Guttridge, D. C., Funkhouser, W. K., and Baldwin Jr, A. S. Selective activation of NF-kappaB subunits in human breast cancer: potential roles for NF-kappaB2/p52 and for Bcl-3, Oncogene. *19:* 1123-1131, 2000.
7. Nozaki, S., Sledge Jr, G. W., and Nakshatri, H. Repression of GADD153/CHOP by NF-kappaB: a possible cellular defense against endoplasmic reticulum stress-induced cell death, Oncogene. *20:* 2178-85., 2001.
8. Romieu-Mourez, R., Landesman-Bollag, E., Seldin, D. C., Traish, A. M., Mercurio, F., and Sonenshein, G. E. Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-kappaB in breast cancer, Cancer Res. *61:* 3810-8., 2001.